ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 310 (2003) 24–28

www.elsevier.com/locate/yviro

Analysis of the herpes simplex virus type 1 UL6 gene in patients with stromal keratitis

Aaron R. Ellison,[a,b] Li Yang,[a] A. Vicky Cevallos,[a] and Todd P. Margolis[a,*]

[a] *The Francis I. Proctor Foundation and Department of Ophthalmology, University of California at San Francisco, San Francisco, CA 94143-0944, USA*

[b] *Bit Tech Inc., Westlake Village, CA 91361, USA*

Received 27 August 2002; accepted 20 November 2002

Abstract

Recent work suggests that herpes simplex virus (HSV) stromal keratitis in the mouse is caused by autoreactive T lymphocytes triggered by a 16 amino acid region of the HSV UL6 protein (aa299–314) (Z.-S. Zhao, F. Granucci, L. Yeh, P. Schaffer, H. Cantor, 1998, Science 279, 1344–1347). In the present study we sought to determine whether genetic variation of this presumed autoreactive UL6 epitope is responsible for different pathogenic patterns of human HSV keratitis. To accomplish this, we sequenced the HSV UL6 gene from ocular isolates of 10 patients with necrotizing stromal keratitis, 7 patients with recurrent epithelial keratitis, and 8 patients with other forms of HSV keratitis. The sequences obtained predicted identical UL6(299–314) epitopes for all 25 viral isolates. Furthermore, the upstream sequence of all isolates was free of insertions, deletions, and stop codons. We conclude that different pathogenic patterns of human HSV keratitis occur independent of genetic variation of the HSV UL6 (299–314) epitope.

Keywords: Herpes simplex virus; Autoimmune response/disease; Immunopathology; Keratitis; Virus infection; Molecular mimicry; UL6

Introduction

Herpes simplex virus (HSV) stromal keratitis is the leading cause of infectious corneal blindness in developed countries. It is typically characterized by recurrent episodes of inflammation resulting in scarring, thinning, and vascularization of the corneal stroma. However, the spectrum of corneal disease caused by HSV is extremely broad and some patients never develop stromal disease despite multiple recurrences of HSV infection of the corneal epithelium (HEK). While most cases of the epithelial form of ocular disease are effectively treated with a short course of a topical or systemic antiviral, topical corticosteroids are the mainstay of therapy for HSV stromal keratitis (HSK). The immunopathological basis of HSK is highlighted by the persistence of neutrophils, lymphocytes, cytokines, and chemokines in diseased corneas long after the clearance of productive viral infection (Streilein et al., 1997).

Mouse models have been studied extensively to investigate the immunopathogenesis of HSK (Thomas and Rouse, 1997), and in one model mice developed autoreactive CD4+ T cells specific for an epitope in immunoglobulin $G2a^b$ ($IgG2a^b$) (Avery et al., 1995). Analysis of the HSV genome revealed a close homology between the $IgG2a^b$ epitope and a 16 amino acid region of the HSV UL6 protein [UL6(299–314)] (Zhao et al., 1998), a component of the viral capsid (Patel and Maclean, 1995). In follow-up studies $CD4^+$ T cells from mice with HSV stromal keratitis were found to be activated by a synthetic 16 amino acid peptide based on UL6(299–314), and mice infected with a replication-defective UL6 deletion mutant failed to develop the corneal inflammation and scarring characteristic of this model (Zhao et al., 1998). Additional studies revealed that a replication-competent viral mutant, engineered with a single amino acid exchange at codon 309 of the HSV UL6 gene, was much less efficient at inducing HSK than wild-type

* Corresponding author. Francis I. Proctor Foundation, 95 Kirkham St., University of California San Francisco, San Francisco, CA 94143-0944. Fax: +1-415-476-0527.

E-mail address: tpms@itsa.ucsf.edu (T.P. Margolis).

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(03)00075-8

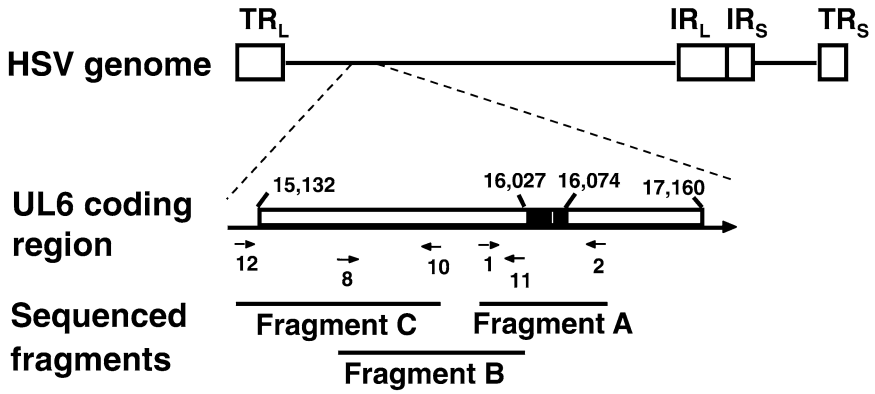

Fig. 1. Schematic representation of the HSV genome showing the position of PCR primers (arrows) and amplified fragments for DNA sequencing (bars). Nucleotide positions of the UL6 coding region are according to McGeoch (1988). The shaded region of the UL6 coding region represents nucleotides 16,027–16,074 and shows the approximate position of the UL6(299–314) peptide. Sequencing fragments A, B, and C represent amplification of nucleotides 15,912–16,190, 15,264–16,001, and 15,057–15,747, respectively. TR_L, long terminal repeat; IR_L, long internal repeat; IR_S, short internal repeat; TR_S, short terminal repeat.

virus (Panoutsakopoulou et al., 2001). The authors of these studies conclude that murine HSK is caused by autoreactive T cells triggered by a small region of the HSV UL6 protein. However, recent work by Rouse and colleagues raises serious questions about the role of molecular mimicry in the pathogenesis of murine HSK and instead provides strong data to support the hypothesis that the immunopathogenesis of HSK is caused primarily by bystander activation of CD4 T cells (Deshpande et al., 2001; Gangappa et al., 1999).

It is tempting to speculate that an immunopathological process, similar to that observed in the mouse, is responsible for HSK in humans. If so, then the wide variation in clinical presentation of HSV keratitis may be due, in part, to genetic variation of the autoreactive UL6(299–314) epitope. The goal of the current study was to determine whether differences in the predicted amino acid sequence of the HSV UL6(299–314) correlate with the presence or absence of stromal disease among patients with recurrent HSV keratitis.

Results

Sequence of UL6 coding region in ocular viral isolates

We amplified and sequenced a fragment of the UL6 gene (Fig. 1, Fragment A) from HSV-1 isolates of 10 patients with HSK, 7 patients with a history of five or more episodes of HEK (but no stromal thinning or vascularization), and 8 patients with HSV keratitis whose clinical history did not fall into either of the above categories. The sequences obtained predicted identical UL6(299–314) epitopes (nucleotide positions 16,027 to 16,074) for all 25 ocular viral isolates as well as for five viral laboratory strains (KOS, ANG, HFEM, McIntyre, and McKrae). Some HSV isolates contained variations at nucleotide positions 15,938 (C → T), 15,951 (C → A), and 16,083 (T → C), but these did not change the predicted amino acid sequence of the UL6 protein.

We next amplified and directly sequenced nucleotides 15,132–15,933 (Fig. 1, Fragments B and C) from all ocular isolates to investigate the possibility that mutations in the upstream portion of the UL6 gene abolish expression of the predicted UL6(299–314) epitope. Sequence comparison of these isolates revealed no evidence of insertions, deletions, or missense mutations that would disrupt the predicted amino acid sequence of the UL6 protein. While several nucleotide variations that alter the predicted UL6 amino acid sequence were found, none were associated with a specific pattern of clinical disease (Table 1).

UL6 RNA expression in infected A549 cells

Since it is possible that defects in the UL6 promoter could abrogate transcription of UL6 RNA and thus prohibit expression of the UL6(299–314) epitope, we next examined expression of UL6 RNA in A549 cells infected with HSV isolates from two eyes with HSK (samples 2 and 3) and eyes with recurrent HEK but no stromal thinning or vascularization (samples 11 and 12). As assayed by semiquantitative RT-PCR, UL6 RNA was present in A549 cells infected with HSV from both groups of patients (Fig. 2). A band of similar size and abundance was observed following infection of cells with the HSV lab strain KOS. These results demonstrate that the UL6 promoter was functional in HSV isolates from both patients with HSK and those with recurrent HEK.

Discussion

HSV infection of the eye can lead to a wide spectrum of HSV keratitis ranging from HEK to HSK. The reason for

Table 1
Predicted UL6 amino acid (aa) sequence variations of HSV isolates as compared to HSV strain 17+[a]

	aa 33[b]	aa 92[c]	aa 96[d]	aa 154[e]	aa 155[f]
Lab strains					
KOS	Ser → Thr	wt	wt	wt	wt
ANG	ND	Gly → Cys	wt	Pro → Leu	Ser → Thr
McKrae, McIntyre, HFEM[g]	ND	wt	wt	wt	wt
HSK isolates					
1, 3	Ser → Thr	wt	wt	wt	wt
2	Ser → Thr	Gly → Cys	Ala → Val	Pro → Leu	Ser → Thr
4, 5, 6, 8	wt	wt	wt	wt	wt
7, 9, 10	wt	Gly → Cys	wt	Pro → Leu	Ser → Thr
HEK isolates					
15, 16, 17	wt	wt	wt	wt	wt
11, 12, 13, 14	Ser → Thr	Gly → Cys	wt	Pro → Leu	Ser → Thr
Other isolates					
18, 19, 20, 21, 22, 25	wt	wt	wt	wt	wt
23, 24	wt	Gly → Cys	wt	Pro → Leu	Ser → Thr

Note. wt, wild-type; ND, not determined; HSK, HSV stromal keratitis; HEK, recurrent HSV epithelial keratitis.
[a] Nucleotide and amino acid positions are according to McGeoch et al. (1988).
[b] $G_{15,176} \rightarrow C$.
[c] $G_{15,405} \rightarrow T$.
[d] $G_{15,418} \rightarrow C$.
[e] $G_{15,592} \rightarrow T$.
[f] $G_{15,594} \rightarrow A$ and $A_{15,596} \rightarrow C$.
[g] HFEM contains the additional variation of Ala → Thr ($G_{15,369} \rightarrow A$) at aa position 80.

this is not known. The recent finding that HSK in the mouse is caused by autoreactive T cells triggered by the HSV UL6(299–314) epitope (Zhao et al., 1998; Panoutsakopoulou et al. 2001) prompted us to study banked HSV1 isolates from patients with HSK and those with recurrent epithelial keratitis but no evidence of stromal necrosis to determine whether variations in the DNA sequence encoding the UL6(299–314) epitope might account for the variable clinical presentation of recurrent HSV keratitis. Analysis of the UL6 gene sequence of ocular viral isolates from 25 patients with varying patterns of recurrent HSV keratitis revealed no polymorphisms that would predict altered expression of the UL6(299–314) epitope. Thus, polymorphisms in the HSV UL6 gene are not responsible for the variable presentation of keratitis in patients with recurrent ocular HSV infection.

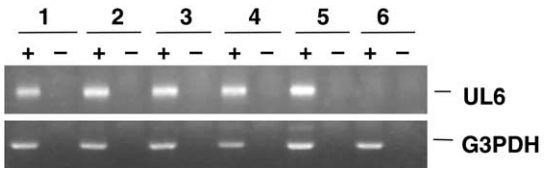

Fig. 2. Analysis of HSV UL6 RNA by RT-PCR. RNA from HSV-infected A549 cells was subjected to RT-PCR analysis as described under Materials and methods. RT-PCR of G3PDH RNA from each set of infected and uninfected cells served as loading control. Reactions were performed with (+) and without (−) reverse transcriptase to verify RNA samples did not contain contaminating DNA. Lanes are as follows: HSV lab strain KOS (lanes 1, + and −), HSV isolate 11 (lanes 2, + and −), HSV isolate 12 (lanes 3, + and −), HSV isolate 3 (lanes 4, + and −), HSV isolate 2 (lanes 5, + and −), and uninfected cells (lanes 6, + and −). HSV ocular isolates 2 and 3 were from patients with herpes simplex stromal keratitis. HSV ocular isolates 11 and 12 were from patients with recurrent herpes simplex epithelial keratitis but no stromal thinning or vascularization.

It is important to point out that our data do not completely rule out a role for the HSV UL6 gene product in the pathogenesis of human HSK. Just as different strains of mice develop either necrotizing or nonnecrotizing keratitis as a consequence of genetic differences in their immune responsiveness to the UL6(299–314) epitope (Zhao et al., 1998), the variable clinical presentation of HSV keratitis in humans may be a consequence of heterogeneity in the immune response. Alternatively, the broad spectrum of HSV keratitis in humans may be a consequence of heterogeneity in host expression of putative corneal autoantigens. Thus, it is still possible that the UL6 protein triggers HSV necrotizing keratitis in humans, but only in genetically predisposed individuals.

In light of the above discussion, demonstration of a role for the UL6 gene product in the pathogenesis of HSK in humans will likely depend upon clinical studies that demonstrate the presence of corneal lymphocytes in patients with HSK that are reactive to both the HSV UL6 protein and the corneal antigens. Although investigators have successfully isolated HSV-specific $CD4^+$ T cells from a small number of human corneas with HSK, these T lymphocytes did not appear to react to either the HSV UL6 protein or the corneal antigens (Verjans et al., 1998, 2000; Koelle et al., 2000). Taken together with our data, this suggests that, unlike the mouse, HSK in humans is not an autoreactive process triggered by a 16 amino acid region of the HSV UL6 protein.

Materials and methods

Laboratory HSV-1 strains

HSV type 1 strain KOS has been propagated in our laboratory for many years. Strains ANG and McKrae were gifts of D. Bloom (University of Florida). Strain HFEM was a gift of B. Mitchell (Baylor School of Medicine). Strain McIntyre was purchased from Advanced Biotechnologies Inc. (Columbia, MD).

Patient diagnosis, HSV specimen collection, and preparation of viral DNA

Banked clinical HSV isolates were obtained from the clinical microbiology lab at the F. I. Proctor Foundation (University of California at San Francisco) and the Campbell Microbiology Laboratory at the University of Pittsburgh Ear and Eye Institute. The definition of HSK varies widely between institutions and among experts in the field. For the purposes of this study, we defined HSK as a keratitis with decreased corneal sensation, thinning, or vascularization of the cornea stroma, and history of a positive ocular viral culture for HSV-1. This study was approved by the UCSF Committee on Human Research and adheres to the Declaration of Helsinki for research involving human subjects.

Viral cultures of corneal scrapings were performed on human bronchial carcinoma (A549) cells (Bartels Inc., Issaquah, WA). All viral isolates were passaged less than two times. Infected cells were lysed at 37°C in a solution containing 10 mM Tris–HCl (pH 8.0), 300 mM sodium acetate, 1 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.2 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO). Cell lysates were frozen at −20°C and centrifuged at 12,000 *g* for 15 min at 4°C to remove SDS–protein complexes. The supernatant fluid containing viral DNA was stored at −20°C.

PCR amplification and sequence analysis of the UL6 gene

The UL6 gene is located between nucleotides 15,132 and 17,160 of the HSV1 genome (McGeoch et al., 1988). Regions of the UL6 gene were amplified by PCR prior to direct sequencing. To accomplish this viral isolates (10–20 μl) were brought to a final volume of 100 μl in a mixture containing 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$, 0.2 mM of each dNTP, 0.1 μM of sense and antisense oligonucleotide primers, 10% DMSO, 0.1% Triton X-100, 100 μg/ml bovine serum albumin and 50 U/ml PFU DNA polymerase (Stratagene, La Jolla, CA). The primers used were UL6-1 (5′-TTG TGG ACC GGG GAC CGT CTG-3′), UL6-2 (5′-GTG GCG CAT GCC CTT CAT GTT G-3′), UL6-8 (5′-GGG CAG CTG GGG TAT ACC GAG-3′), UL6-11 (5′-CGC GTG TTC CCG GAG GGA GTG-3′), UL6-10 (5′-CCG CGG CCT CCG CGA CCG TG-3′), and UL6-12 (5′- GTC CCC GGG TTG CTG AAG GTG-3′). Thermocycling conditions were as follows: 94°C for 2 min followed by 37 cycles of 94°C for 36 s, 55°C for 2 min, and 72°C for 2.5 min. To validate successful amplification by PCR, an aliquot of the PCR product was electrophoresed on a 2% agarose gel containing 0.25 μg/ml ethidium bromide. The remaining sample was purified with Centricon-40 columns (Princeton Separations, Adelphia, NJ) for direct sequencing using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin–Elmer, Foster City, CA).

RNA preparation from infected cells

A549 cells were infected with HSV and grown at 37°C until 100% of the monolayer exhibited cytopathic effect. At this stage, growth media was removed; cells were washed with phosphate-buffered saline (pH 7.4), and total RNA was prepared using TRIzol reagent (Life Technologies, Gaithersburg, MD). RNA samples were treated with a solution containing 0.8 U/ml RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.7 U/ml RNase Inhibitor (Roche Molecular Biochemicals) at 37°C for 60 min. RNA was then purified by phenol–chloroform extraction, precipitated with ethanol, and resuspended in diethyl pyrocarbonate-treated H_2O.

Reverse transcription (RT)-PCR

RNA (1.5 μg) was reverse transcribed in a 20-μl mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 2 U/μl RNase Inhibitor (Roche Molecular Biochemicals), 2.5 μM random hexamer (Pharmacia, Piscataway, NJ), and 10 U/μl Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL, Grand Island, NY). Samples were incubated at 23°C for 10 min, 37°C for 45 min, 95°C for 5 min, and 18°C for 1 min. An aliquot representing 1/25th of the resultant first-strand cDNA was amplified by PCR as described above. For the HSV-1 UL6 gene, primers UL6-1 and UL6-2 were used. For the cellular glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, primers G3PDH-1 (5′-ACC ACA GTC CAT GCC ATC AC-3′) and G3PDH-2 (5′-TCC ACC ACC CTG TTG CTG TA-3′) were used.

Acknowledgments

We thank Y. Jerold Gordon and Regis Kowalski for clinical isolates of HSV-1 from the Campbell Microbiology lab, David Bloom for HSV-1 strains ANG and McKrae, and Brad Mitchell for HSV-1 strain HFEM. This work was supported by National Institutes of Health Grants EY10008 and EY02162, a Research to Prevent Blindness Lew Was-

serman Merit Award (New York, NY), and the Ralph and Sophie Heintz Laboratory Fund (San Francisco, CA).

References

Avery, A.C., Zhao, Z-S., Rodriguez, A., Bikoff, E.K., Soheilian, M., Foster, C.S., Cantor, H., 1995. Resistance to herpes stromal keratitis conferred by an IgG2a-derived peptide. Nature 376, 431–434.

Deshpande, S.P., Lee, S., Zheng, M., Song, B., Knipe, D., Kapp, J.A., Rouse, B.T., 2001. Herpes simplex virus-induced keratitis: evaluation of the role of molecular mimicry in lesion pathogenesis. J. Virol. 75, 3077–3088.

Gangappa, S., Deshpande, P., Rouse, B.T., 1999. Bystander activation of CD4 T cells can represent an exclusive means of immunopathology in a virus infection. Eur. J. Immunol. 29, 3674–3682.

Koelle, D.M., Reymond, S.N., Chen, H., Kwok, W.W., McClurkan, C., Gyaltsong, T., Petersdorf, E.W., Rotkis, W., Taley, A.R., Harrison, D.A., 2000. Tegument-specific, virus reactive CD4 T cells localize to the cornea in herpes simplex virus interstitial keratitis in humans. J. Virol. 74, 10930–10938.

McGeoch, D.J., Dalrymple, M.A., Davidson, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E., Taylor, P., 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69, 1531–1574.

Panoutsakopoulou, V., Sanchirico, M.E., Huster, K.M., Jansson, M., Granucci, F., Shim, D.J., Wucherpfenig, K.W., Cantor, H., 2001. Analysis of the relationship between viral infection and autoimmune disease. Immunity 15, 137–147.

Patel, A.H., Maclean, J.B., 1995. The product of the UL6 gene of herpes simplex virus type 1 is associated with virus capsids. Virology 206, 465–478.

Streilein, J.W., Dana, M.R., Ksander, B.R., 1997. Immunity causing blindness: five paths to herpes stromal keratitis. Immunol. Today 18, 443–449.

Thomas, J., Rouse, B.T., 1997. Immunopathogenesis of herpetic ocular disease. Immunol. Res. 16, 375–386.

Verjans, G.M.G.M., Remeijer, L., Mooy, C.M., Osterhaus, A.D.M.E., 2000. HSV-specific T cells infiltrate the cornea of patients with herpetic stromal keratitis: no evidence for autoreactive T cells. Invest. Ophthal. Vis. Sci. 41, 2607–2612.

Verjans, G.M.G.M., Remeijer, L., van Binnendijk, R.S., Cornelissen, J.G.C., Volker-Dieben, H.J., Baarsma, S.G., Osterhaus, A.D.M.E., 1998. Identification and characterization of herpes simplex virus-specific CD4+ T cells in corneas of herpetic stromal keratitis patients. J. Infect. Dis. 177, 484–488.

Zhao, Z-S., Granucci, F., Yeh, L., Schaffer, P.A., Cantor, H., 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. Science 279, 1344–1347.